AD-A136 611 RAPID CHROMATOGRAPHIC ANALYSIS OF ENZYMES AND OTHER PROTEINS (RYCHLA CHRO..(U) DEFENCE RESEARCH INFORMATION CENTRE ORPINGTON (ENGLAND) O MIKES NOV 83 DRIC-T-7049 1/1

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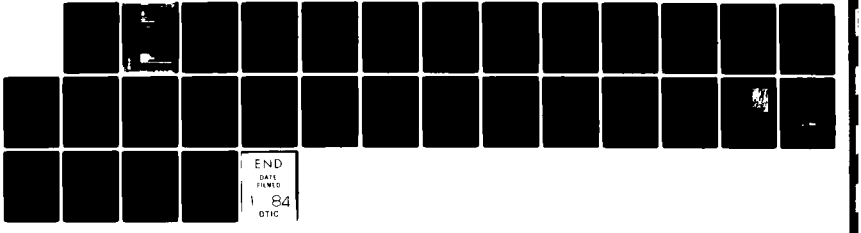

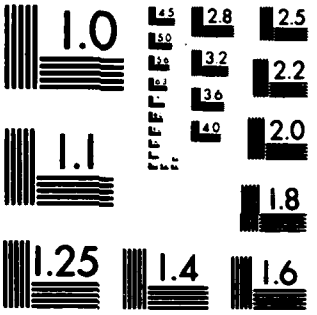

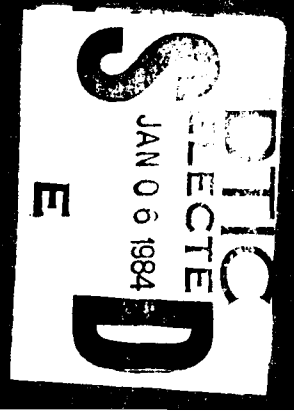

Rapid chromatographic analysis of enzymes and other proteins

Chemicke listy, 76,(1982) 59 - 79

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(from Czech)

C o n t e n t

1. Introduction

Bary L.Karger, reviewing the "Third International Symposium on Column Liquid Chromatography " (held 27 - 30. Sept. 1977, in Salzburg, Austria), wrote[0]: "Surprisingly, there were no lectures on the separation of proteins using bonded phases in liquid chromatography ... High performance separation of proteins certainly remains one of the major challanges " . To-day, in 1981, it is possible to cite examples of how this challenge had been answered within a few years. The aim of this article is to review contemporary rapid chromatographic column methods which reduce the time required for the separation of enzymes and their mixtures with proteins from a number of hours (and sometimes several days) to a few tens of minutes (and sometimes even to a few minutes). The article seeks not only to comment on the methods directly available for the food technology, research, manufacture and application of technical enzymes. It also seeks to stimulate their wide use and to perfect the methods developed in other fields of biochemistry for purposes of food analysis. That is why it tries to sketch further development rather than review the methods used up to now in the analysis of foodstuffs.

High-performance (high-pressure) liquid chromatography (HPLC) and medium pressure liquid chromatography (MPLC) were or are being,

increasingly accepted into a number of branches of chemical research and for analytical control of the manufacture, especially where gas chromatography cannot be used. Frequently they even successfully compete with the latter. Its fundamental principles have already been developed satisfactorily and described not only in numerous review articles, but also in a number of monographs[2-6]. They are also rapidly introduced into the field of biochemistry[7-10]. The way they have been used in the field of food-analysis is known not only from literature data, such as studies[11,12], but also from plenary lectures, papers and posters of the 1st European Congress on Food Chemistry [13,14]. It is understandable that there were endeavours to use these rapid methods, which were found suitable for substances with low and medium molecular mass, also for the separation of high-molecular biopolymers, particularly polypeptides and proteins[15-18]. However, the rapid separation of peptides is not the subject of this paper.

2. LC and HPLC (MPLC) of biopolymers

Conventional liquid column chromatography (LC) of proteins was initially considered to be a difficult problem, since there were no suitable chromatographic packings available. Various inorganic substances showed strong irreversible sorption and organic ion-exchangers with an aromatic matrix frequently denatured proteins by a strong hydrophobic interaction. Only the slightly acid acrylate and methacrylate cation exchangers (of the Amberlite IRC 50-type) could be used for these tasks[19]; however, these were microporous materials and, therefore, only groups on the surface of particles were functional. Only Peterson and Sober[20-22], with their ion-exchange derivatives of cellulose, Porath and Flodin[23], with crosslinked polydextran, and Porath and Lindner[24], with ion-exchange derivatives of polydextran, prepared hydrophilic and, at the same time, macroporous packings which are eminently suitable for the chromatography of all types of biopolymers.

They were successfully used in tens of thousands of studies and the writer of this article is of the opinion that this methodological contribution by the above authors for the development of modern biochemistry, molecular biology and fields based on them has not been appreciated sufficiently. Quite recently, these packings have also been supplemented by crosslinked agarose and its ion-exchange derivatives.

However, all these materials, so important for the conventional column chromatography of biopolymers, are very soft and do not permit the use of higher pressures. Therefore, they are not suitable for purposes of HPLC and MPLC. A further disadvantage is that their polysaccharide matrices are reactive to certain enzymes and can therefore be liable to attack by microorganisms. Ion-exchange derivatives of of polydextran also markedly change the volume of the bed as a function of the ionic strength. Therefore, in line with the development of HPLC, new materials were sought which would also be suitable for the pressure chromatography of biopolymers. They must not only be macroporous and sufficiently hydrophilic, but also hard so as to resist to pressures in the column. They must be spheres of uniform size, chemically resistant, stable in aqueous solutions in a wide pH range, must have a constant volume independent of the ionic strength and resist organic solvents. They must not be split by enzymes and must be resistant to the action of microorganisms. It is not easy to find packings which satisfy all these requirements.

3. Packings for HPLC and MPLC of proteins

Schechter[26] was the pioneer in the HPLC of proteins; in 1973, he chromatographed the carboxylic acid synthetase and other proteins on "Porous silica gel 1000" or on "Porasil DX". He had previously deactivated these packings with Carbowax-20 M so as to suppress an undesired irreversible sorption. Coupek et al.[27] synthesised, in

1972, the macroreticular hydrophilic glycolmethacrylate gel, Spheron[28], the structure of which is represented in Fig. 1. This material also satisfactorily resists organic solvents[29]. In 1975, Hikes et al.[30] prepared, by the modification of its hydroxy groups, ion-exchange derivatives suitable for the HPLC and MPLC of biopolymers[16]; weakly, medium and strongly acid and also weakly and strongly basic ion exchangers were prepared and tested for the rapid chromatography of proteins[31-35].

A very significant contribution for the HPLC of proteins was the investigation carried out in 1975 in the laboratory of Regnier et al.[17,36-40]. The glass with controlled porosity[41-43] developed by Haller and microporous silica gels, such as those of Zorbax, Porasil, Partisil and LiChrospher[44], show undesired interactions with proteins (partially irreversible adsorption, cationic sorption and anionic exclusion of proteins). Threfore, in the above laboratory, methods for surface modification were developed, by which the outer and extensive inner surface of inorganic particles (spheres) was enveloped by a hydrophobic layer, the so-called glycophase. This is essentially glycerol conveniently bound by a hydroxy group through propylsilane over the entire surface of the particle. The macroporous glass thus modified (Glycophase-CPG) or silica gel (e.g. SynChropak GPC) acquire a neutral hydrophilic surface binding the water and can be used for steric separation chromatography of proteins and other biopolymers under HPLC conditions, see study[43]. The LiChrosorb DIOL[46] packing has a similar structure and use. Engelhardt and Mathes[47] modified chromatographic packings with N-acetylaminopropylsilane for the same purpose.

One of the glycerol hydroxy groups of the glycophase layer can be iogen substituted and so rigid microporous ion exchangers can be prepared with a hydrophilic matrix suitable for ion-exchange chromatography of biopolymers by the HPLC methods[37-40]. In this way were prepared weakly basic DEAE, weakly acid CM, strongly acid SP and

strongly basic QAE Glycophases. Ion exchanger derivatives for the HPLC of proteins were however also prepared by enveloping the surface of microparticulate spheres with a continuous layer of polyethyleneimine which was not covalently bound to their surface; in this way was prepared SymChrom AX which has the properties of a weakly basic anion exchanger with higher nominal capacity.

An independent chapter in the development of the HPLC of proteins and peptides is provided by reversed phase chromatography, RPC[7], based on hydrophobic interactions[49]. Certain hydrophilic packings show a certain degree of hydrophobicity and can be used directly for hydrophobic chromatography of proteins; such is e.g. Spheron[50]. In other cases, it was necessary to make hydrophilic macroporous polysacharide packings artificially hydrophobic by the introduction of hydrocarbon chains[51,52]. For purposes of the RP-HPLC of peptides and proteins, inorganic packings were developed: porous silica gels with their whole surface modified with these hydrocarbon chains: (C_2), (C_8) and (C_{18}), i.e. the so-called "ethyl, octyl, and octadecyl-bonded phases " . The chains are bonded most easily via monochlorodimethylalkylsilanes[53]. So were modified e.g. pelliculate Corasil or the entirely porous LiChrosorb. From aqueous solutions, proteins or peptides become sorbed on to hydrocarbon chains by hydrophobic interactions and, at higher ionic strength, become "salted" on to the hydrophobic surface. By the addition of less polar solvents (e.g. alcohols or acrylonitrile) to the mobile phase, they are gradually eluted. For the separation of proteins and peptides with higher M_r value C_8 - bonded phase[54] was found suitable with n-propanol as a regulator of the polarity of the mobile phase, or C_{18}-bonded phase[55] with isopropanol or 2-methoxyethanol as a regulator. These packings are also suitable for ionic pairing agents. Recently, Lewis et al.[56] developed C_8 RPC packings with sufficiently high porosity which they prepared from LiChrosphere Si 500 (pores 50 nm) or from Vydac (pores 33 nm); besides packings with bonded octyl groups, packings

with bonded cyanopropyl or diphenyl groups were also prepared. The higher porosity of supports improves the chromatography of proteins by $M_r > 50\ 000$.

Besides size exclusion, ion-exchange, hydrophobic and reverse phase chromatography, further rapid chromatographic methods were developed for the separation of proteins based on principles which are not identical with the above. Rubinstein[54] describes the so-called "normal phase chromatography ", using a support which has been made hydrophilic, i.e. LiChrosorb DIOL, where he attains separation using a decreasing concentration of n-propanol. Hashimoto, Fukano et al.[57,58] mention new Japanese packings, the so-called TSK-Gely SW, destined for the gel HPLC in aqueous media; these are packings based on silica gel modified by a hitherto undescribed method by organic substances also non-specified which may obviously affect[17] the course of chromatography. On the other hand, Mituzani and Mituzani[59] showed that anionic silane groups on the inner surface of non-modified glass with controlled porosity (CPG) can sorb proteins similarly to cation exchanger and thus make chromatographic separation possible. Affinitive chromatography[60] was also developed into the HPLC form. Ohlson et al.[61] used as packings adenosine-monophosphate bonded on silica gel for the rapid separation of proteins and immobilised anti-bodies from albumin antiserum for the rapid separation of serumalbumin from other components of the serum. Turkova et al.[62] used as packing high performance liquid affinitive chromatography (HPLAC) Separon-E-ε-aminocyproyl-L-Phe-D--PheOMet. Essentially, Separon E is Spheron surface-modified by the introduction of epoxide groups by the reaction with epichlorohydrin.

Table I lists a survey of various types of commercially available chromatographic packings for HPLC which were used for the rapid chromatography of enzymes and other proteins and their fragments. In study[63] is given a characterisation of certain commercial packings for SEC (Size exclusion chromatography).

4. Examples for the chromatographic separation of proteins, their fragments and enzymes

4.1. Gel permeation and steric (size) exclusion chromatography

One of the most common principles of the separation of biopolymers is fractionation depending on the size of the molecules. On xerogels (i.e. supports with a crosslinked matrix, the size of macropores of which greatly changes with the degree of swelling, e.g.polydextrans), this principle is designated as gel-permeation chromatography (GPC). For aerogels (i.e. packings with constant size of macropores even after drying, e.g. in the case of glass with controlled porosity), it is more appropriate for this principle to use the designation of steric (size) exclusion chromatography (SEC). However, these differences in terminology are being consistently dispensed with.

Using a relatively rapid method, Haller et al.[64] chromatographed immunoglobulin concentrate from human serum on non-substituted glass with controlled porosity (CPG) as far back as 1969, but did not call their method HPLC. Eltekov et al.[65], in 1972,similarly investigated the chromatography of proteins on Silichrom C-80 which they modified with γ-aminopropyltriethoxysilane. Schechter[2] in a pinoneering study on the HPLC of proteins, using SEC, separated, on deactivated "Porous silica gel 1000 ", catalase, thyroglobulin and Blue dextran in 20 min and, in another experiment on an identical packing, in a similarly short period, he isolated an active microbial fatty acid synthetase ($M = 2.5 - 3 \times 10^6$) from contaminating proteins. On deactivated Porasil FX, he also isolated other proteins (e.g. β-hydroxydecanoylthioesterdehydrase). Regnier and Noel[36] studied extracts of various proteins from glyceropropylsilane-bonded phases (Glycophase G/CPG) and, besides proteins (e.g.sera), they also chromatographed nucleic acids and dextrans. On non-substituted Spheron 1000 (a hybrid aerogel/xerogel, see[28]), in 1975, Vondruska et al[66] were the first to separate proteins; at that time, the incomplete fractionation was attributed to GPC, but later Strop et al.[67]

demonstrated, in more complete separations of proteins, that the main separating principle on this non-modified packing was hydrophobic interactions. Chang et al.[38] separated on microparticulate-bonded hydrophilic phases (Glycophase G/CPG, Glycophase G/LiChrospher Si-100 and Glycophase G/Partisil PXS) proteins from natural mixtures (e.g. from liver homogenates) and tried to carry out a very rapid SEC of albumin and cytochrome c in 2 min. Persiani et al.[45], using the GPC method, chromatographed, on glycerol-CPG, industrial protein glues (both pure and after infection by bacteria) and also checked the linear dependence of log M_r of proteins on the elution volume on GPC for these materials. Fischer et al.[67] separated, using the SEC method, on "Glycophase G/CPG ",insulin, glucagon and somastatin, Niemann et al.[68] a partially purified complement D. Koumeliotis and Unger[69] chromatographed, on LiChrosorb DIOL, a number of proteins from cytochrome c (M_r = 12000) to ferritin (M_r = 540000) and found that this packing is suitable for M_r 10000-100000. Gruber et al.[70] proved the possibility of determining the M_r of polypeptides and proteins with the aid of SEC on SynChropak GPC-100 beginning with vasopressin and ending with cattle serumalbumin and also separated several extracts of biological origin. For comments on GPC (SEC) of proteins on SynChropak GPC and on other packings see[17]. Rapid SEC on "single protein " and "dual protein columns I 125" as an alternative to the conventional GPC and gel electrophoresis are given by Rittinghaus and Franzen[71]; they separated ferritin (M_r = 540000), cattle serumalbumin (M_r = 67000), egg albumin (M_r = 45000), myoglobin (M_r = 17000), ribonuclease A (M_r = 13700) and cytochrome c (M_r = 12500) during 25 min.

A large group of studies on GPC and SEC of proteins with the aid of new Japanese packings of TSK-SW gels has already been published by Japanese[57,58,72-77] and also by other authors[78]. In study[57], the GPC of 14 peptides and proteins was tested, beginning with human fibrinogen

(M_r = 340000) and ending with diglycin (M_r = 132). Wehr and Abbot[78] give a Table of data suitable for the study of SEC (review of M_r and of the length of the main gyration axis for selected proteins and viruses as well as an evaluation of various packings); they separated 5 proteins from cytochrome c to α-globulin and also nucleic acids in the range of M_r 13500 -340000 with the aid of TSK-2000 and 3000 SW columns and MicroPack MAX 500. In study[73] were separated plasma proteins, study[74] is devoted to the investigation of the separating range and separating effectiveness on various TSK-SW gels and study[75] describes the purification of enzymes (β-galactosidase from bacterial cells and commercial urease); a single GPC resulted in a 15-fold purification. For the purpose of studying how to make the determination of M_r more accurate, in study[76], the chromatography was investigated of proteins in a range of M_r between 50000 and 300000 on various TSK-SW gels in solutions of sodium dodecylsulphate and in studies[72,77] in 6 M quanidinehydrochloride; Nobuo Ui[72] describes a rapid and relatively accurate determination of M_r of proteins after fission of disulphide bridges by reduction and substitution of SH groups.

4.2. (Ionex) Ion-exchanger chromatography

Ion-exchange chromatography is one of the most significant processes for the separation of proteins. Compared with GPC and SEC, its advantage is the much higher separation-capacity of ion exchangers for proteins; compared with the former mentioned principles, it permits a higher loading of the columns for the same sized bed. Further, the possibility of using gradients (ionic strength and pH) provides separation facilities. In our laboratory, as far back as 1975, we tried to use ion-exchange derivatives of Spheron for the rapid chromatography of biopolymers, including a number of proteins[30]; we carried out comparative chromatography of egg proteins on CM-cellulose and CM-Spheron, on phospho-Spheron, we carried out fractionation of human serumalbumin, of bovine chymotripsin and chicken lysozyme, of

A and B chains of insulin and also of human plasma which was also chromatographed on DEAE-Spheron. On S-Spheron was carried out the rapid chromatography of commercial glucose-oxidase (on a 0.8 x 25 cm column, 6 ml fractions at intervals of 90 sec). High-molecular deoxyribonucleic acids from calf-thymus were also separated, as were oligonucleotides from the partial DNA hydrolysate of Bacillus subtilis. Study[79] was devoted to the analysis and to the preparative reversible sorption of commercial enzymes (protease from Aspergillus sojae on CM and DEAE-Spheron, glucose-oxidase and pectolytic enzyme on DEAE-Spheron); see also Fig. 2. In the framework of studies on DEAE-Spheron[32] and on CM-Spheron[33] were separated lysozyme, chymotripsin, serumalbumin and egg proteins. Results obtained by the rapid chromatography of proteins and of their fragments (e.g. bromocyanated fragments of serumalbumin on CM-Spheron) on Spheron ion exchangers are the subject of a review report[16]. The detailed chromatographic separation of pectolytic enzymes Rohament P and Petinex Ultra on all available types of Spheron ion exchangers is described in study[80].

A different series of reports on the rapid ion-exchange-chromatography of proteins was also independently developed in American laboratories from 1975 onwards. Kudirka et al.[81] investigated the separation of isoenzymes of creatinekinase on "Vydac pellicular anion-exchanger". Chang et al.[37] chromatographed human serum on a support with bonded polyethyleneimine phase and proteolytic enzymes and the homogenate of rat liver on packing with bonded DEAE-phase (CPG and Porasil C). Chang et al.[38] describe the separation of human serous proteins, of various haemoglobins, alkaline phosphatase, isoenzymes of creatino - phosphokinase and lactatedehydrogenase (LDH) on DEAE-Glycophase/CPG. Kudirka et al.[82] also investigated the chromatography of LDH-isoenzymes on DEAE-Glycophase/CPG. In study[39], Chang et al. describe the chromatography on all types of Glycophase/CPG ion exchangers of commercial trypsin-inhibitor from soya beans on CM, commercial

chymotrypsinogen on SP and commercial trypsin and creatinephospho-kinase isoenzymes on DEAE, as well as a mixture of proteins on QAE derivatives. Bissett[83] used chromatography to separate a cellulolytic complex from Trichoderma resei on DEAE-Glycophase/CPG which he prepared according to[38]. Gooding et al.[84] separated on SynChropak AX 300 haemoglobin variants of human blood. Alpert and Regnier[85] developed porous microparticulate anion exchanger packing *, particularly SynChropak AX and used them for the rapid anion-exchange chromatography of human serum, LDH-isoenzymes from rat kidneys and hexokinase from rat livers as well as the chromatography of nucleotides.

4.3. Hydrophobic reversed phase chromatography

A "pure" hydrophobic interaction chromatography on non-substituted Spheron is described by Strop et al.[30]. At a higher ionic strength, a number of proteins "becomes salted" on to the Spheron matrix and is freed at lower ionic strength. The elution is made easier by the addition of alcohols (e.g. tert.-butanol) which reduce the polarity of the mobile phase. Authors investigated these processes for the separation of human serumalbumin, chymotrypsinogen and lysozyme, human serous proteins, raw pig pancreatic α-amylase and for peptides from a tryptic hydrolysate of lysozyme. Subsequently, Strop and Cechova[86] used these methods for the separation of difficultly separable α, β and ψ-trypsins. The hydrophobic interaction property of Spheron is greatly suppressed by ionogenic substitution[32]. O'Hare and Nice[87], using hydrophobic interaction methods in a HPLC arrangement, separated a number of physiologically active peptides and also certain proteins (insulin, cytochrome c, lysozyme, myoglobin) on silica gel with alkylsilane-bonded phases (Hypersil ODS, Partisil ODS, Spherisorb ODS, Nucleosil 5-C_{18}, LiChrosorb RP-18 (and RP-8), Zorbax-C_8). By similar methods, Nice et al.[88] isolated proteins from endocrinic and para-endocrinic tissues and cells.

* During editing, we received a reprint of a study by Vanecek and Regnier (113) dealing with a similar subject

HPLC on reverse phase packings is often used for the rapid separation of peptides and it is only now being introduced for the separation of proteins. The problems are associated with the fact that organic solvents used for it tend to denature the proteins (enzymes). The designation "reversed phase chromatography" (RPC) is essentially the result of the original idea from the early years of the development of separation chromatography, when the polar aqueous phase was commonly considered to be fixed and the non-polar organic phase to be mobile. Now, on hydrophilic macroporous supports (mostly porous silica gel), hydrocarbon chains are covalently-bonded. From polar aqueous solutions, by hydrophobic interactions, molecules of biopolymers are bonded reversibly by their hydrophobic portions on to these. By the addition of organic non-polar solvents (e.g.acetonitrile), they are gradually liberated into the mobile phase. Research aims primarily at finding an effective composition of the mobile phases for a selective desorption which would, at the sametime, prevent denaturation of biopolymers. Thus Honch and Dehnen[55] investigated the chromatography of 8 proteins (from insulin to ferritin) on Nucleosil 10 C-18 in an acid phosphate buffer, using a mixture of isopropanol and 2-methoxyethanol as a regulator of polarity; they found that, up to high M_r of 450000, the RPC is effective and highly reproducible. Congote et al.[89] separated by rapid RPC human globule chains on Bondapak C_{18}, with the use of acetonitrile and trifluoroacetic acid. Dinner and Lorenz[90] separated various insulins on LiChrosorb RP-8 by isocratic elution (acetonitrile-0.2 M ammonium sulphate). Petrides et al.[91] separated mutation variants of haemoglobin chains on octadecasilyl stable phases, with the use of propanol and pyridine formate. Lewis et al.[56], during the development of new supports for RPC (see Chapter 3), separated by chromatography tyrosinase (M_r = 128000), α_1 -chains of collagen (M_r = 95000) and also other subunits of collagen, bovine serumalbumin (M_r= 68000) and cytochrome c (M_r =12500) .

One of the now-developing branches of RPC is ion-pair reversed phase HPLC. In fact, the elution of hydrophobic dissolved substances from the bed of the reversed phase depends not only on the reduction of the polarity of the mobile phase, which is the usual working method for RPC; it can also be attained by increasing the polarity of the dissolved substance which is hydrophobically-bonded on the support. This can be achieved by ion-pairing, with the use of a counter-ion or haeteron in the mobile phase. For instance, the dissolved substance forms an ion complex with the haeteron which is easily soluble in water. During chromatography, this complex behaves as a single substance : the haeteron "entrains" the dissolved substance with it into the mobile phase. Conversely, with hydrophobic ion-pairing agents, the dissolved substance can be more strongly bound to the bed. By a suitable choice of such complexes and by using various haeterons according to the nature of the dissolved substance, their retention or elution can be largely influenced. For that purpose, one can use a number of complex-forming substances and wetting agents including inorganic ions. These methods are developed for the contemporary rapid chromatography of peptides and are now also being investigated for the separation of proteins. A group of New Zealand researchers has been largely responsible for their successful development, see review by Hearn and Hancock[15,92]. Besides a number of peptides, various insulins and their chains, proinsulin, bromocyanated fragments of haemoglobins and enzymatic hydrolysates of proteins were separated. Packings known from RPC were found suitable, such as Bondapack C_{18} or C_{18}-Sep-Pak. Rivier[93] showed that triethylammonium phosphate is a very suitable agent for the elution of peptides and lower proteins (insulin, cytochrome c) by ion-pairs.

5. Instrumentation for the HPLC (μPLC) of proteins and post-column enzyme detection

Although experience is available for the technical realisation for the HPLC of lower molecular substances[4] and even though there are

on the market the first effective commercial analysers of proteins (e.g. Waters Protein Separation System PSS)[71], an entirely satisfactory universal instrumentation for the HPLC of proteins is still outstanding. Table II(which does not claim to be complete), arranged in the order of references, informs the reader of the commercial devices used in the studies quoted. In a number of cases, the authors themselves assembled or modified the chromatographic device from accessible components. As long as the researcher is satisfied with medium-pressure chromatography (MPLC), components for this can be used from the analyser of amino acids or sugars and through-flow recording spectrophotometers, e.g.[33,50,86]. The instrumentation of the hydrophobic reverse phase chromatography of proteins does not present fundamental problems since, in most cases , non-corrosive liquids are used. Problems arise when full automation of ion-exchange chromatography is attempted: including regeneration, cycling and the equilibration of ion exchangers in the column. For instance, Spheron ion exchangers are very stable and permit cycling on 2M NaOH and 2M HCl frits[16]. The non-rusting pumps used[33], and also the columns for HPLC in organic solvents, regretfully liberated traces of ions of heavy metals even into a 0.1M ammonium formate buffer of pH 3.5 . This is a drawback during the chromatographing of certain enzymes. The need to remove, at least occasionally, the packing from the column for extended cycling and equilibration on the frit, reduces the gain from the significant reduction in time for the chromatography itself, made possible by the development of the new ion exchangers. Therefore, a universally usable protein-analyser is still waiting for its development.

A significant problem facing food analysis is the specific detection of enzymes after HPLC of analysed specimens. It can be solved by the routine analysis of removed fractions (e.g.[79-82]), or possibly by using the Technicon-Autoanalyser[97] or some other suitable arrangement[98]. A significant chapter in this complex of problems was opened up by Chang

et al.[38] by their post-column enzyme detector. This makes possible the through-flow specific detection of a single type of enzyme in which the effluent from the fractionation column is mixed with a suitable substrate, the mixture is passed through a heat-treated reaction column (packed with non-porous microspheres) and the product is detected by UV-detection at a wavelength at which the absorption by proteins is not observed (an example is given in Fig. 3), or a fluorescence-detection is carried out. From that time, post-column enzyme detectors underwent intensive development and found use especially in clinical diagnostics for the specific detection of isoenzymes[99,101,107]. The system with the reactive packed column[38] was further developed in the studies of Schlabach et al.[99,101,102], whilst Schroeder et al.[100], Fulton et al.[104] and Schwabach et al.[105] developed the principle of the through-flow of effluent with the substrate through a heat-treated reaction capillary. The effect of the background was solved by deducting the absorbency of the non-reacted mixture from the reacted[103,104]. For the realisation of certain detection reactions, it is necessary to pump-in certain further (frequently expensive) enzymes, together with the substrate, especially in the case of multi-stage reactions (see biochemical principles in Table III). This is a disadvantage. Therefore, detectors were developed with immobilised auxiliary enzymes[101]. The specific "on-line" enzyme detection primarily developed for clinical diagnostics has[106,107] however a very promising future for the further development of food chemistry.

6. Possibility of applying the HPLC (MLC) of enzymes and other proteins in food chemistry and manufacture

Post-column enzyme detectors are able "to see" in complex mixtures of proteins (e.g. serum) only the desired type of enzyme. This is very important during the analysis of isoenzymes for medical diagnostics[106,107], since changes in mutual proportions of isoenzymes (which can be rapidly separated from each other by the HPLC irrespective of the presence of

other proteins) indicate a diseased state. A relatively perfect and rapid separation of isoenzymes was developed for lactatedehydrogenase [38,99,100-102,104,105], creatinephosphokinase[38,99,101,102], alkaline phosphatase and hexokinase[101] and arylsulphatase[106]. Other isoenzymes, e.g. human amylases, were separated as well as by electrophoretic methods, by affinative chromatography also[108]. However, principles were proposed for "on-line" detection for HPLC and other important enzymes, e.g. certain proteases and also proteins containing SH groups[99]. All these findings developed for purposes of clinical biochemistry are valuable for food chemistry, since not only isoenzymes, but various multiple forms of enzymes of other types are encountered in food products and commercial enzymes. For instance Aoshima[109] demonstrated by the HPLC method the multiple forms of lipoxygenase-1 of soya beans and, in our laboratories, during the study of commercial preparations, we found multiple forms of certain pectolytic[80] *and cellulolytic*[110] enzymes (figs. 4 and 5) .

The possibility of the rapid specific detection of multiple forms of enzymes is of great importance for the control of the quality of manufacturing processes and for storage. It is well-known that, on the initial limited proteolysis, many enzymes do not lose their activity but change their electrophoretic or chromatographic mobility. And so a careful control of the products with respect to the mutual relation of multiple forms of enzymes opens the way to the "biochemical diagnostics" of large areas of food technology. It makes it possible to observe rapidly, specifically and sensitively mutual transitions between various forms of enzymes as a function of the method of processing and storage and thus to indicate eventually certain undesired processes, as happens in clinical diagnostics (pathological changes). Similarly, differences in quality during fermentation in the manufacture of commercial enzymes, they can be very rapidly noticed by an "on-line" detector.

However, the HPLC of proteins on its own, without the post-column enzyme detection, has great significance for food control and manufacture. First of all, it permits the rapid analytical differentiation of the quality of various proteins. Just as the chromatographic profiles for serous proteins can differ in patients with various illnesses, so conclusions can be drawn for various food products and processes involving proteins. It becomes possible e.g. to differentiate between raw materials, intermediate products and products according to origin, to estimate their age and to detect various contaminations, etc. The great advantage is the speed of all these analyses which is one to two orders higher than the conventional LC which takes several hours and even days. This speed permits the continuous observation of the most varied fermentation processes during the manufacture of foodstuffs and their immediate regulation on the basis of rapidly obtained data. This was not possible before, since only finished products were analysed. An example of the rapid observation of the kinetics of laboratory fermentation of deoxyribonucleic acid by deoxyribonuclease is given in study[38]. Similarly, the manufacture of commercial enzymes and other bioproducts during the cultivation of microbes in large fermentors can now be controlled with the aid of HPLC when, "once and for all", they are identified in advance and individual peaks are calibrated.

The application of HPLC is also tested on a preparative scale[111]. In certain cases, it is already in technical use during the manufacture of polypeptide and protein preparations for medical purposes. The future application of HPLC of biopolymers in the biochemical and fermentation industries was discussed at the Conference in Bratislava[112].

The aim of this article is to draw the attention of readers to these rapidly developing disciplines and to offer an introductory essential base for further study and also for independent investigations.

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Received for publication: 15.4.1981

O. Mikeš (*Institute of Organic Chemistry and Biochemistry of the Czechoslovak Academy of Sciences, Prague*). **Rapid Chromatographic Analysis of Enzymes and Other Proteins.**

Theses of this lecture were presented at the 1st European Congress of Food Chemistry EURO FOOD CHEM 1 Vienna, Febr. 17.—20., 1981; cf. Ernährung/Nutrition *5* (1981) 88—98. The review presents fundamental data and a survey of references to the lecture on chromatographic column methods for rapid separation of enzymes and other proteins. These methods were worked out in other fields yet they offer many important applications in food chemistry. The introduction deals with the rapid orientation of the development of HPLC toward biochemistry and outlines the problems of the LC of biopolymers. Next follows a survey of collumn packings for the HPLC of proteins and of rapid separations of enzymes and other proteins by gel-permeation (or size/ /steric'-exclusion), ion-exchange, hydrophobic, reversed-phase, and ion-pair reversed phase chromatography; their principles are briefly described. The present state of instrumentation of the HPLC (MPLC) of proteins is given and the principles of specific post-column enzyme detectors are explained. The review is concluded by a discussion of the possibilities of the application of the HPLC (MPLC) of enzymes and other proteins in food chemistry.

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5. v nemcine = in German

13. Special issue of the Journal "Ernahrung, Nutrition, 5, No.2, 1981

63. v tisku = awaiting publication

Označení	Výrobce (dodavatel)	Reference
Bondapak C_{18}	Waters Associates, Inc Milford, Mass., 01757 USA	15, 89
Spheron 300, Spheron 1000, Spheronové měniče iontů	Lachema, Brno (Chemapol Ltd., Praha) Československo	16, 30 až 35, 50, 66, 79, 80, 86
Porous silica Gel-1000 (Woelm EM-Gel, Type SI) (desaktivovany Carbowaxem-20M) Porasil-Dx (desaktivovany Carbowaxem-20M) Controlled Pore Glass CPG (modifikovaný), Glycophase CPG	Chromatec, Inc.	26
Porasil C	Waters Associates, Milford, Mass., USA	26
Partisil PXS 10 25 column (modifikovany) Lichrospher Si 1000 (modifikovaný) Vydac FP, Vydac silica supports: Vydac pellicular anion exchanger	Corning Glass, Medfield, Mass., USA (Pierce Chem. Co. Rockford, Ill., USA) Electronucleonics, Inc., Fairfield, N. J., USA	45
	Auspec, Ann Arbor, Mich., USA	37
	Whatman, Clifton, N. J., USA	38
	EM Labs, Elmsford, N. Y., USA	38
SynChropack AX	The Separation Groups, 8738 Oakwood Ave., Hesperia, Calif. 92 345, USA	48, 56, 81
LiChrosorb, LiChrospher Chromosorb LC-6 Spherisorb Alumina	SynChrom, Inc., P. O. Box 110, Linden, Indiana 47 955, USA	48, 84
	EM Labs, Elmsford, N. Y., USA	48
	Johns-Manvill, Denver, Colo., USA	48
	Phase Separations, Ltd., Queensferry, Flintshire, Gt. Britain	48
Nucleosil 5-C_{18}; 10-C_{18} EM Lichrosphere Si-500 EM Lichrosorb RP-8 TSK-PW gels, TSK-SW gels, TSK-SWG columns	Machery-Nagel, Duren, GFR	55, 87
	Ace Scientific, Linden, N. J., USA	56
	Toyo Soda Manufact. Co, Ltd. Tonada, Shinnanyo City, Yamaguchi Pref., Japan	57, 58, 72 - 78
Separon HEMA (Spheron), Separon F	Laboratorní přístroje, n. p. 162 03 Praha, Československo	62
I-125 Protein Analysis Columns Lichrosorb DIOL columns MicroPak MAX 500	Waters Associates, Milford, Mass., USA	68, 71
	Du Pont de Nemours, Bad Nauheim, GFR	69
	Varian Aerograph, Walnut Creek, Calif., USA	78
DEAE-Glycophase CPG-250	Corning Glass Works, Corning Biological Products Department Medfield, Mass., 02052 USA	82
Hypersil-ODS, -SAS, -APS Spherisorb-ODS	Shendon, Runcorn, Gt. Britain	87, 88
	Phase Separations, Queensferry, Gt. Britain	87
Lichrosorb RP-18, RP-8 Zorbax-C_8 Partisil 10-ODS Supelcosil C_{18} RP-18 columns	Merck, Darmstadt, GFR	87, 90
	Du Pont, Hitchin, Gt. Britain	87
	Whatman, Maidstone, Gt. Britain	88
	Supelco, Bellefonte, Pa., USA	91
	Brown Lee Laboratories, Berkeley, Calif., USA	91

I. Commercial packings for the HPLC (MPLC) of enzymes and other proteins (can be used as such or after modification)

Headings: Designation; Manufacturer (Supplier); References

Entries: a) Spheron ion exchangers.... Czechoslovakia
b) (Deactivated with Carbowax-20 M)
c) (Modified)

Označení	Výrobce (dodavatel)	Reference
Chromatograf pro střední tlaky, sestavený z proporcionálního programového mikročerpadla 68 005, kolon (0,8 — 25 cm) a náhradních součástí k aminokyselinovému analyzátoru, tandemového systému dvou průtokových UV-analyzátorů (A_{285}, A_{254}) a jímače frakcí spojeného se dvěma linearními zapisovači	Vývojové dílny ČSAV, Laboratorní přístroje n. p., Československo	16, 33
Liquid Chromatograph LC 2200	Chromatec, Inc.	26
Gradient marker device (modifikovaný)	Chromatec, Inc.	26
Precision Sampling Model 420 inlet	Precision Sampling, Baton Rouge, La., USA	37
Isco Model 384 Pumping system	Instrument specialities, Lincoln, Neb., USA	37, 38
Micromeritics Model 7000 Liquid chromatograph; 254-nm detector; Model 705 column packer	Micromeritics, Norcross, Ga., USA	38, 83
Perkin-Elmer LC-55 detector, nebo Model NFC 254 UV detector	Perkin-Elmer, Norwalk, Conn., USA	38, 39
Disc. model sample injection valve	Disc. Instrument Inc., Costa Mesa, Calif., USA	39
Waters Associates Model 202 Liquid Chromatograph	Waters Associates, Milford, Mass., USA	45
Constametric I a II G system	Laboratory Data Control, Riviera Beach, Fla., USA	48
Rhydone 7120 sample Injecotr	Anspec Co, Ann-Arbor, Mich., USA	48
Perkin-Elmer LC-55 variable-wave length detector	Perkin-Elmer, Norwalk, Conn., USA	48
Aminco Fluoro-Monitor	American Instrument Co, Silver, Md., USA	48
Micromeritics Column Packer, Model 705	Micromeritics, Norcross, Ga., USA	48
Knauer 2050 RI detector	Knauer, GFR	50
Spektrální UV analyzátor (typ UVM-4); proporcionální mikročerpadlo-68 005; průtoková fotocela (typ DUV, 254 nm)	Vývojové dílny ČSAV	50, 86
Waters Associates liquid chromatograph; M 6000 solvent delivery system; Model 660 solvent programer	Waters Associates, Milford, Mass., USA nebo Königstein, GFR	55, 68
U6K Septumless universal injector system	Waters Associates, Milford, Mass., USA	55, 68, 81
Rheodyne injector	Rheodyne, Berkeley, Calif., USA	56
USG (0-4000psi) pressure gauge	Navtec Industries, Hicksville, N. Y., USA	56
Chrontrol unit	Lindburg Enterprises, San Diego, Calif., USA	56
Milton Roy Simplex Minipumps	Laboratory Data Control, Riviera Beach, Fla., USA	56, 70
Liquid chromatograph HLC-802 UR nebo Model HLC-803	Toyo Soda Manufact. Co., Ltd., Tonda, Shinnanyo City, Yamaguchi Prefect., Japan	57, 58 73—78
Model UA-5 absorbance monitor	Isco, Lincoln, Neb., USA	67
Model 6000A pumps, Model 660 solvent programer, U6K injector	Waters Associates, Milford, Mass., USA	67, 90
Chromatograph Du Pont Model 830 instrument	Du Pont de Nemours, Bad Nauheim, GFR	69, 87, 88
7000psi Injector valve	Valveo Instrument Co., Houston, Texas, USA	70
Protein Separation System (PSS)	Waters Associates, Milford, Mass., USA	71
Waters Model 204 Liquid Chromatograph	Waters Associates, Milford, Mass., USA	71
Hitachi 635 high-pressure liquid chromatograph, Hitachi 034 double beam effluent monitor (průtoková kyveta s 1 cm světelnou stopou)	Hitachi Perkin-Elmer, Hitachi Ltd., Tokyo, Japan	72
Varian Model 5020 gradient HPLC system s Variachrom variable wavelength absorbance detector	Varian, Palo Alto, Calif., USA	78, 90
Model 820 liquid chromatograph	Du Pont de Nemours et Co., Inc., Instrument Product Division, Wilmington, Del. 19 898, USA	81, 82
16-Port valve (No ASCN-16-HPa-C20)	Valveo Instrument Co., Houston, Tex. 77 024, USA	82
Reciprocing piston pump, Model No 721-33 Solvent Delivery System, 16—320 ml	Laboratory Data Control, Division of Milton Roy Co., Riviera Beach, Fla., 33 404 USA	82
Model 204 590 six-port sample-loop valve	Du Pont de Nemours et Co., Inc., Instrument Product Division, Wilmington, Del. 19 898 USA	82
Autoanalyzer II single channel colorimeter	Technicon Instruments Corp., Tarrytown, N. Y. 10 591, USA	82
Varian Variscan, Model 635, UV-VIS spectrophotometer	Varian, Palo Alto, Calif., USA	83

Označení	Výrobce (dodavatel)	Reference
Model 837 variable wavelength spectrophotometer	Du Pont, Hitchin, Gt. Britain	87, 88
Schoeffel Model 970 fluorimeter		87
838 Programable Gradient Module a Microprocessor Controled Spectra Physics SP 800	Spectra Physics, Santa Clara, Calif., USA	88
Rheodyne sampling valve	Rheodyne, Berkeley, Calif., USA	90
Model 110 A pumps a Microprocessor gradient control unit	Altex, Berkeley, Calif., USA	91

II. Instrumentation for the HPLC (HTLC) of proteins

Headings : Identical with those in Table I

Entries : a) Chromatograph for medium pressures, assembled from proportional programmed micropump 68 005, column (0.8 x 25 cm) and spare components for the aminoacid analyser, tandem system of two through-flow analysers (A_{285} , A_{254}) and fraction collector coupled with two linear recorders

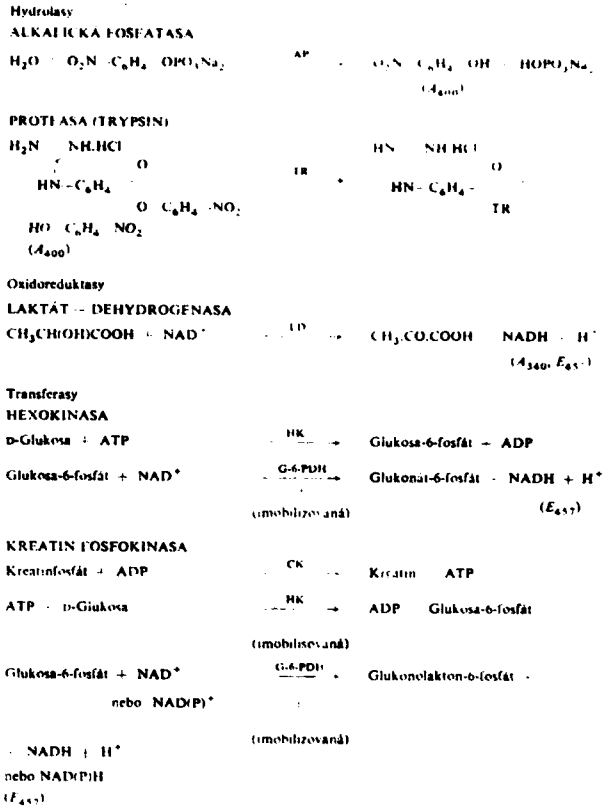
b) modifikovaný = modified

c) nebo = or

d) Spectral UV analyser (type UVM-4); proportional micropump 68 005; through-flow photocell (type DUV, 254 nm)

e) Through-flow cell with 1 cm light track

f) and



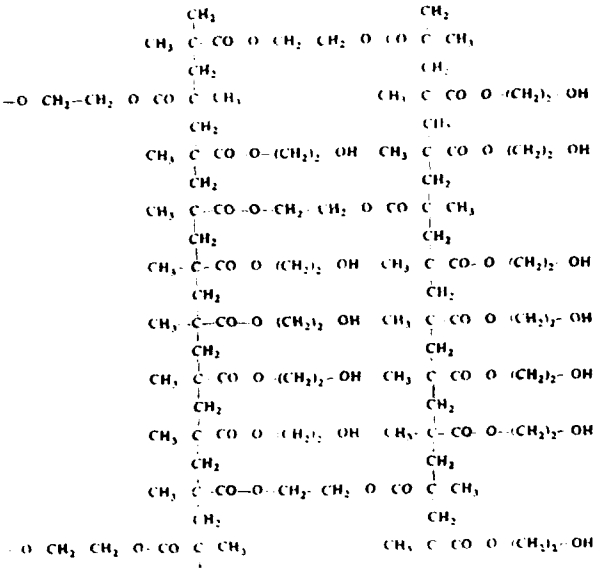
III. Biochemical principles of post-column enzyme detectors[99,101]

In spite of different spelling, entries appear to be easily understood, except for the following:

alkalicka = alkaline

imobilisovana = immobilised

nebo = or

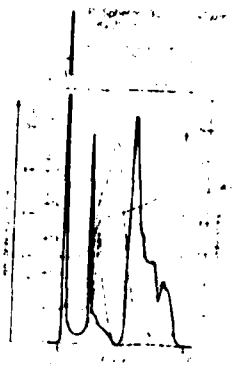


1. Microstructure and macrostructure of Spheron spheres[30,31]

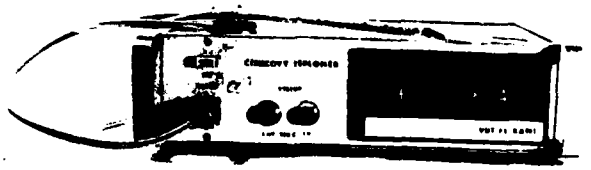
   A) Glycolmethacrylate macroreticular very densely crosslinked (and therefore mechanically strong) gel is separated using a special suspension by copolymerisation initially in the form of submicroscopic drops, so-called microspheres. These agglomerate already during the polymerisation into larger spheres (macrospheres) of about 10 - 100 micron dia. The developed macropores (the most frequent dia is 250 or 370 A) form an extensive inner surface (about 100 m^2/g) with numerous hydroxy groups suitable for ionogenic substitution or affinant bonding. The extremely chemically-stable repeating structural unit reminiscent of esters of pivalic acid, $(CH_3)_3C.COO.Et$, ensures the chemical resistance of the matrix

   a) Macrosphere; b) Macropore; c) Micropore; d) Microsphere

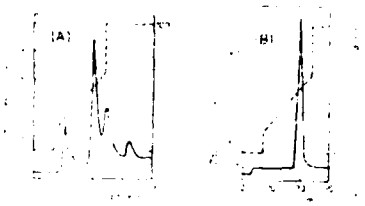
   B) On the electronic microphotograph of the section through a Spheron sphere, agglomerates of microspheres and the cavities between these, which form the macropores, are observable



2. Chromatography of an old partly-deactivated commercial preparation of a microbial α-amylase (e.g. subtilis) on a Phospho-Spheron 300 column, using an aqueous-alcoholic solution for the elution of hydrophobically-bonded contaminant with strong absorbency.
Effluents: A – 0.05M NH_4OH + HCOOH, pH 4.0;
B – 0.25M NH_4OH + CH_3COOH, pH 6.0;
C – 0.5M NH_4OH + CH_3COOH, pH 6.0 ;
D – C + t-BuOH, 1:1 (v/v); E – H_2O+t-BuOH, 1:1 (v/v);
F – H_2O; G – 2M NaCl
The peak for the residue of the original active enzyme is marked by a broken line
a) All gradients are linear; b) Detector record;
c) Conductivity



3. Digital laboratory thermometer
a) Input; b) Output

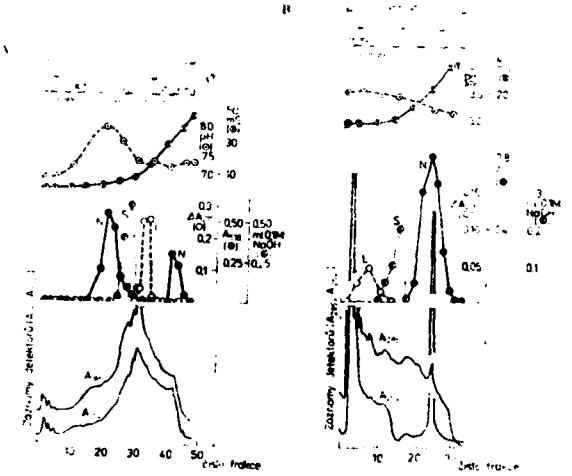


3. Post-column enzyme detection. According to Chang et al.[38]

A) xample of the HPLC of a commercial calf intestinal phosphatase on a 50 x 0. cm column from DEAE-Glyconhase/CPG in Tris-buffer, pH 8 with gradient of NaCl with the usual UV detection at 280 nm

B) Analogous chromatography with specific post-column enzyme detection of alkaline phosphatase with pumping-in a substrate of p-nitrophenylphosphate to the effluent and UV detection at 410 nm after passing through the reaction column; there was no observable widening of the peak. p-Nitrophenol formed is detected

a) Detection recorder; b) of solvent; c) Time

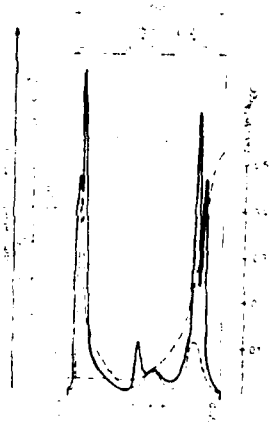


4. Examples of the separation of commercial (technical) pectolytic enzymes on 20 x 0.8 cm Spheron ion exchanger columns, with the use of gradients of ionic strength. According to[20]

A) Pectinex ultra on Spheron DEAE-1000 in Tris-HCl buffer of pH 7. L pectinlyase, N endo-D-galacturonanase, S pectinesterase

B) Rohment P on Spheron-1000 in sodium formate of pH 3.5

a) Linear gradient; b) No. of fraction;
c) Detector records



5. Chromatography of a commercial cellulolytic enzyme system (after the cultivation of Trichoderma viride) on a 20 x 0.8 cm column from DEAE-Spheron 300, 20 - 40 µm. According to[11]

Citrate buffer of pH 5, gradients of ionic strength. Fractions of 2.4 ml taken at intervals of 62 sec. The so-called "filter-paper " activity is marked by broken lines

a) Detector record; b) Conductivity; c) Activity; d) Fraction

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9999OOON	Chemicke listy, 76, (1982) 59 - 79
5a. Sponsoring Agency's Code (if known) 7221OOON	6a. Sponsoring Agency (Contract Authority) Name and Location Procurement Exec., Min. of Defence Defence Res.Info.Centre, UK.

7. Title

RAPID CHROMATOGRAPHIC ANALYSIS OF ENZYMES AND OTHER PROTEINS

7a. Title in Foreign Language (in the case of translations)

RYCHLA CHROMATOGRAFICKA ANALYZA ENZYMU A JINYCH BILKOVIN

7b. Presented at (for conference papers). Title, place and date of conference

8. Author 1.Surname, initials	9a Author 2	9b Authors 3, 4...	10. Date	pp	ref
Mikes, O.			11.1983	27	113

11. Contract Number	12. Period	13. Project	14. Other References

15. Distribution statement

15. Descriptors (or keywords)

Liquid chromatography, Chromatographic analysis, Enzymes, Proteins, Separation, Food analysis.

continue on separate piece of paper if necessary

Abstract Reviews contemporary rapid chromatographic column methods which reduce the time required for the separation of enzymes and their mixtures with proteins from a number of hours to a few tens of minutes. Emphasis is placed on high-performance (high-pressure) liquid chromatography (HPLC) and medium pressure liquid chromatography (MPLC). Methods directly available for the food technology, research, manufacture and application of technical enzymes are discussed.

RS

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